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Cytomegalovirus-Specific T Cells Isolated by IFN-[gamma] Secretion Assay Do Not Induce Significant Graft-Versus-Host Reactions In Vitro.

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TITLE

Cytomegalovirus specific T-cells isolated by IFN-ϒ secretion assay do not induce significant graft-versus-host reactions *in-vitro*

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ABBREVATIONS

CBA, cytometric bead array

CFSE, carboxyfluorescein succinimidyl ester

CMV, cytomegalovirus

CTL, cytotoxic T lymphocytes

$^{+}$donor, seropositive donor

EBV, Epstein-Barr virus

FACS, fluorescence-activated cell sorting

GvH, graft-*versus*-host

GvHD, graft-*versus*-host disease

GvHR, graft-*versus*-host reaction

HLA, human leukocyte antigen

Hour, h

HSCT, haematopoietic stem cell transplantation

IE-1, immediate early protein-1

IFN-Ɣ, interferon-gamma

IFN-Ɣ secretion assay, gamma catch

ml, millilitres

MLR, mixed lymphocytes reaction

PBMC, peripheral blood mononuclear cell

PHA, phytohaemagglutinin

pp65, phosphoprotein 65

SDC, supplementary digital content

SEM, standard error of the mean

ABSTRACT

Background

Graft-versus-host disease (GvHD) remains a serious concern for patients undergoing anti-viral cellular therapy. Despite the major improvements in cellular immunotherapy, the immunogenicity of virus-specific T-cells has not yet been fully defined. This present study aims to examine how cytomegalovirus (CMV)-specific cytotoxic T-lymphocytes (CTLs) respond to allogeneic antigen stimulation and whether they give rise to GvHD-target-tissue damage.

Methods

CMV-CTLs were isolated by the interferon-gamma (IFN-ϒ) secretion assay (gamma-catch) from healthy seropositive volunteers and expanded *in-vitro*. The levels of intracellular IFN-ϒ, cytotoxic activity, IFN-ϒ and granzyme B secretion and CD25 expression were measured using flow cytometry (FACS). The ability of CMV-CTLs to induce GvHD-target-tissue damage was evaluated using the human *in-vitro* skin explant assay (skin explant assay).

Results

CMV-CTLs responded specifically to CMV-phosphoprotein 65 (pp65)-stimulation by secreting IFN-ϒ and killing virus peptide loaded autologous phytohaemagglutinin-blasts (PHA-blasts). Compared to unselected-PBMCs, CMV-CTLs induced significantly less severe cutaneous GvH-tissue damage. This observation coincided with low levels of CD25 expression, as well as IFN-ϒ and granzyme B secretion following allogeneic antigen stimulation in both the mixed lymphocyte reaction (MLR) and in the skin explant assay.

Conclusions

CMV-CTLs isolated by the IFN-Ɣ secretion assay from HLA-unmatched healthy donors exhibited a high level of anti-CMV potency without inducing significant cutaneous GvH-tissue damage *in-vitro*. This finding provides novel evidence supporting the safe use of *in-vitro* expanded CMV-CTLs as an anti-viral therapy in transplant patients with refractory CMV infections.

INTRODUCTION

Haematopoietic stem cell transplantation (HSCT) represents a treatment for malignant and non-malignant blood disorders. Despite considerable success, application is limited due to graft-*versus*-host disease (GvHD)[1]. Conditioning regimen before transplant, increased immunosuppression to prevent/treat GvHD and other risk factors (age, human leukocyte antigen (HLA)-disparity, previous infections) delay the post-transplant immune reconstitution leading to latent-virus reactivation, adding to morbidity and mortality[2,3].

CMV is a common β-herpesvirus, between 30% and 90% in the occidental population[4,5]. In immunocompetent individuals, CMV infection is unproblematic. However it can reactivate in 20-30% of immunocompromised patients undergoing HSCT hence affecting the overall survival[6]. CMV-serostatus of donor-recipient pairs is one of the major risk factors for CMV reactivation or *de novo* infection. The worst case scenario of CMV reactivation/infection is when seropositive patients are transplanted from seronegative donors or *viceversa*[7].

Pre-emptive therapy with anti-viral drugs, for viral infections in post-HSCT patients, is often not sufficient due to drug-resistance[8] and toxicity[9,10]. Adoptive immuno-therapy for the restoration of anti-viral immunity, pioneered by Riddell and colleagues[11], has been considered an alternative treatment for patients with refractory viral infections after failure of standard therapies. Several reports have demonstrated that adoptive virus-CTLs can successfully reconstitute cellular anti-viral immunity by reducing the viral load and even the severity of virus disease in HLA-matched and -mismatched transplant recipients[12,13]. Despite these promising results, *de novo* or exacerbated

GvHD remains a potential risk associated with the adoptive cellular therapy in (un)related mismatched transplant recipients.

In the last two decades, the purity of isolated virus-specific CTLs has improved encouragingly[14,15,16], thus potentially minimizing the risk of alloreactive GvHD. Most clinical trials have reported the reconstitution of anti-viral immunity after adoptive transfer of CMV-CTLs without major complications, although small subgroups of patients had onset or aggravation of pre-existing GvHD[17,18,19,20,21]. Surprisingly, no correlation between the cell dose and the success of anti-viral immune-reconstitution has been observed[22,20]. Very low doses of anti-Epstein-Barr virus (EBV) specific T-cells, such as 148 $CD3^+$ T-cell/kg, have been reported to efficiently clear the viremia[23]. These results suggest that increasing the purity of anti-viral T-cells and reducing the cell-dose might abrogate the onset of GvHD while restoring the anti-viral immunity.

Despite the low incidence of GvHD after adoptive virus-CTL therapy, several *in-vitro* studies have reported a broad allo-HLA-cross-reactivity of memory virus-CTLs[24,25,26]. Amir and colleagues reported that virus-CTL lines or clones (CMV, EBV, varicella zoster and influenza virus) were cross-reactive with a large panel of EBV-transformed B-cell lines transduced with allo-HLA molecules. In addition, virus-specific T-cells showed cross-reactivity against allo-HLA antigens expressed by normal B-cells, T-cells, dendritic or endothelial cells and fibroblasts, suggesting a potential *in-vivo* alloreactivity[27,26]. It has also been suggested that cross-reactivity may be tissue-specific due to differences in self-peptide antigen presentation by the allo-HLA-molecules on specific target-organs[24,28].

Cross-reactivity of virus-CTLs for transfusion has also been reported by Melenhorst and colleagues, but patients who received adoptive transfusion of *in-vitro* cross-reactive bi-virus-specific T-cell lines had no clinical GvHD, suggesting no correlation between *in-vitro* alloreactivity and the clinical outcome[29]. In addition, virus-specific T-cells isolated by gamma-catch, together with a short-period of *in-vitro* expansion, showed less alloreactivity in the MLR when compared to unmanipulated donor lymphocytes[30,31]. Collectively these data may support the low immunogenicity of adoptive viral-specific T-cells and a disassociation between *in-vitro* tests and the clinical GvHD outcome.

The present study investigates whether CMV-CTLs, isolated by gamma-catch, are able to mediate GvH-tissue damage using an unique human *in-vitro* GvHD skin explant model which has been used to predict GvHD in HLA-matched siblings[32]. The skin explant assay has been successfully used to demonstrate the specificity of minor histocompatibility antigen-specific cytotoxic T-cells[33] and more recently to investigate the mechanisms of action of regulatory T-cells for the prevention of GvHD[34,35]. Here we provide the first *in-vitro* evidence demonstrating the severity of pathological changes in GvHD-target-tissue mediated by CMV-CTLs.

MATERIALS AND METHODS

Samples

Sixty millilitres (ml) of peripheral blood was collected from CMV-seropositive donors (CMV^+donor) for the isolation of CMV-CTLs. Blood and skin for the skin explant assay was obtained from healthy volunteers. All donors were recruited by a research nurse with informed consent and approval by the Local Research Ethics Committee.

Isolation of CMV-CTLs by gamma-catch

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. PBMCs were stimulated *in-vitro* with the pp65 protein of human CMV (PepTivator® CMV pp65-premium grade, Miltenyi Biotec, Bergisch Gladbach, Germany) at a concentration of 1µg/ml and incubated for 4 hours (h) at 37°C and 5% CO_2. Cells were then collected and processed as per manufacturer's instructions (Large Scale IFN-ϒ Secretion Assay Enrichment Kit, Miltenyi). Briefly, *in-vitro* stimulated PBMCs were incubated for 5 minutes with IFN-ϒ Catchmatrix Reagent and then incubated for 45 minutes at 37°C in a humidified incubator under slow rotation to allow the secretion of IFN-ϒ. Subsequently, cells were treated with anti-IFN-ϒ micro-beads for magnetic separation by using a manual MidiMACS® Separator and LS separation column (Miltenyi). Unstimulated cells served as the negative controls.

FACS

The purity, specificity, functionality and potential alloreactivity of isolated and *in-vitro* expanded CMV-CTLs were assessed by FACS. Cells were stained following standard protocols and manufacturer's instructions (Inside Stain Kit, Miltenyi). FACS

data were acquired on the BD FACS Canto II cytometer. Where possible, at least 100,000 cells were recorded and analysed by FlowJo v10 software (Tree Start). The following antibodies were used (all obtained from BD Biosciences, Oxford, UK, unless otherwise indicated): CD45 (APC), CD3 (V450), CD4 (FITC), CD8 (PercpCy5.5), CD14 (APC-CyTm7), IFN-ϒ (PE) (Miltenyi), CD4 (APC), CD25 (PE), CFSE (carboxyfluorescein-succinimidyl-ester, Life Technologies, Paisley, UK) and LIVE/DEAD® Aqua Stain (Life Technologies).

In-vitro expansion of CMV-CTLs

CMV-CTLs were expanded *in-vitro* for 2-4 weeks. All eluted cells were co-cultured with irradiated autologous feeder cells (20Gy) at a ratio of 1:100 and 250 IU/ml of IL-2 (Miltenyi). The medium was replaced every 2-3 days. At the end of each week, cells were counted and subcultured as appropriate. After expansion, IL-2 was removed and cells were frozen until required for the experiments.

The intracellular IFN-Y expression in expanded CMV-CTLs

CMV-CTLs were plated at a density of $1.5x10^6$/150 µl of medium and re-stimulated with pp65 (1µg/ml) for 2h. Unstimulated cells were used as the control. Brefaldin A (1µg/ml, BioLegend, London, UK) was then added and cells were incubated for an additional 4h. After a total incubation time of 6h, cells were harvested, attained and data were acquired by FACS.

Cytotoxicity Assay

CTL activity was evaluated by a CFSE-based cytotoxicity assay, slightly modified from that previously described[36]. Briefly, blast target cells were generated by stimulation of autologous PBMCs with 5µg/ml of phytohaemagglutinin (PHA, Sigma-

Aldrich, Dorset, UK) for 5 days. Medium was then replenished every 2-3 days for 2 weeks and IL-2 (100 IU/ml) was added. $CD4^+$ PHA-blasts were isolated by magnetic separation (Miltenyi) and stimulated overnight with pp65 (5µg/ml), whereas unstimulated PHA-blasts were the unloaded control. PHA-blasts (loaded and unloaded) were then stained with CFSE 3 µM and plated at a density of $5x10^5$ cells/ml per well. CTLs were incubated with PHA-blasts at 20:1, 5:1 and 1:1 ratios for 4h at 37°C, 5% CO_2. Cells were then collected and stained with appropriate antibodies and viability dye. Stained cells were transferred into TruCountTM Tubes (BD) containing a fixed amount of beads to allow the quantitative analyses of the T-cell populations and data were acquired by FACS. Each experiment was performed in duplicate or triplicate and the percentage of specific lyses was calculated as follows: % of lysis= 100-(absolute no. of viable $CFSE^+$ target cells (t=x)/absolute no. of viable $CFSE^+$ target T-cells (t=0))*100 where t=x is the number of viable $CFSE^+$ target cells in the presence of effector cells and t=0 is the number of viable $CFSE^+$ target T-cells in the absence of effector cells.

MLR and expression of CD25 activation marker

CMV-CTLs or unselected PBMCs from the same CMV^+donor were incubated in a MLR with an equal number of previously irradiated (20Gy) allogeneic PBMCs from a HLA-mismatched donor (stimulator), at a density of $2x10^6$ cells/ml, in T25-flasks. After 7 days of co-culture, cells were collected and the CD25 activation marker was investigated by FACS.

Skin explant assay

The skin explant assay was originally described by Vogelsang and colleagues[37] and subsequently revised by Dickinson et al.[32] We investigated the potential of CMV-

CTLs to elicit GvHR in HLA-mismatched individuals and compared the results to those obtained with unselected-PBMCs from the same CMV^{+}donor. Briefly, virus-CTLs or unselected-PBMCs were incubated in a MLR with an identical number of irradiated stimulator PBMCs. After 7 days, CMV-CTLs and unselected-PBMCs were washed and co-incubated with the recipient's skin-tissue. 72h later the skin biopsies were collected and assessed for histopathological damage. The grading was performed according to Lerner's criteria[38] (I-IV): grade-I GvHR: mild vacuolization of basal cells (background); grade-II-IV reactions: positive for GvHR, giving rise to diffused vacuolization of basal cells with scattered dyskeratotic bodies in the epidermal layer (grade-II), subepidermal cleft formation (grade-III) and complete separation of the epidermal and dermal layer (grade-IV) (Figure 1).

HLA genotyping for virus epitopes

The genotyping for 12 potential virus-HLA restrictions (A*01:01, A*02:01, A*11:01, A*24:02; B*07:02, B*44:03, B*44:02, B*40:01, B*35:01, B*35:02, DRB1*01:01) was performed by PCR-SSOP at the NHS Blood and Transplant Centre, Newcastle Upon Tyne.

IFN-Ɣ and granzyme B detection

The levels of IFN-Ɣ and granzyme B in the supernatants of the skin explant assay were quantified using the Cytometric Bead Array (CBA) Kit (BD) following the manufacturer's instructions. Data were analysed with FCAP Array Software (BD).

Statistical analysis

Statistical analyses were performed using paired and unpaired Student's t-test (GraphPad Prism 5). The results were reported as median or mean ±standard error

of the mean (SEM). Correlation studies were performed using Spearman's test (non-parametric correlation) and Chi-Square Fisher's test (SPSS-Statistics v.21). Differences were considered significant with a $P \leqslant 0.05$.

RESULTS

Isolation and *in-vitro* expansion of CMV-CTLs

CMV-CTLs were successfully isolated from 20 healthy CMV^+donor by gamma-catch. For each isolation experiment, unstimulated-PBMCs were included as a negative control and FACS analysis was used to assess the purity of the separation. The pre-selection frequency of CMV-CTLs secreting IFN-ϒ after pp65-antigen specific stimulation was 1.60% (mean; ±0.58%) for $CD3^+$ T-cells, 0.78% (mean; ±0.19%) for $CD4^+$ T-cells and 2.10% (mean; ±0.92%) for $CD8^+$ T-cells (data not shown). After enrichment, a mean yield of $3.66x10^5$ (±0.96) cells was obtained (data not shown) and the purity of CMV-CTLs was increased to 69.07% (±5.96%) for $CD3^+$, 67.28% (±5.79%) for $CD4^+$ and 68.02% (±7.07%) for $CD8^+$ T-cells (Figure 2-A+supplemental digital data (SDC)-Figure 1) with no difference in the ratio between $CD4^+$ or $CD8^+$ T-cell populations (data not shown). To investigate the potential allo-reactivity of CMV-CTLs by the skin explant assay, the cells were expanded *in-vitro* for 2 to 4 weeks giving rise to a 235.80 (±82.43) fold increase (Figure 2-B). The $CD8^+$ population expanded to a significantly greater extent (P=0.004). The total number of $CD4^+$ and $CD8^+$ CMV-CTLs after expansion was $3.65x10^6$ ($\pm1.25x10^6$) and $1.55x10^7$ ($\pm3.51x10^6$), respectively (Figure 2-C).

In-vitro expanded CMV-CTLs exhibited antigen-specific cytotoxicity

Following *in-vitro* expansion, the percentage of T-cells responding to CMVpp65 stimulation remained similar, except for the $CD4^+$ T-cell population. After short term re-stimulation with the pp65-antigen, the frequency of CMV-CTLs measured by intracellular IFN-ϒ staining was 52.98% (±6.17%) for the $CD3^+$ and 66.86% (±5.18%)

for the $CD8^+$, while the $CD4^+$ T-cells decreased to 32.81% (±5.27) (Figure 3-A+SDC-Figure 2).

The capacity of CMV-CTLs to kill target-cells presenting the viral antigen was further demonstrated by a CFSE-based cytotoxicity assay, where CTLs were incubated together with autologous PHA-blasts, with or without pp65. Expanded CMV-CTLs could specifically lyse autologous PHA-blasts presenting the pp65-antigen in a dose dependent manner (*P*=0.0079, *P*=0.0459 and *P*=0.07 for effector:target ratios of 20:1, 5:1 and 1:1) (Figure 3-B+SDC-Figure 3).

CMV-CTLs showed a low level of activation by allogeneic-antigens

CMV-CTLs were examined for CD25 expression after stimulation with allogeneic-PBMCs in a MLR (n=5). CMV-CTLs showed a significantly reduced CD25 allo-activation compared to the respective unselected-PBMCs from the same CMV^+donors in all T-cell populations (*P*<0.0001, *P*<0.004 and *P*<0.0002 for $CD3^+$, $CD4^+$ and $CD8^+$ T-cells, respectively) (Figure 4+SDC-Figure 4).

CMV-CTLs showed reduced *in-vitro* GvHR

CMV-CTLs and the allo-unselected-PBMCs from the same individual were tested as a third party donor at a low ($5x10^5$) and a high ($1x10^6$) cell dose in the skin explant assay for GvHR activity (n=9). As a positive control, skin was co-cultured with allo-unselected-PBMCs ($5x10^5$) from HLA-unmatched $CMV^-$$^+$donor. This led to grade-II and -III GvHR. In contrast, using the corresponding CMV-CTLs ($5x10^5$), all skin sections demonstrated grade-I background GvHR. The difference in the histopathology grade between allo-unselected-PBMCs and CMV-CTLs was statistically significant (*P*<0.0006). Skin co-cultured with the high cell dose ($1x10^6$) of

allo-unselected-PBMCs resulted in grade-II, -III and -IV GvHR in the skin explant assays, whereas the corresponding high cell dose of CMV-CTLs showed significantly less severe GvHR (P<0.0001) with 7/9 CMV-CTLs showing a grade-I background reaction and two CMV-specific T-cell lines (donor 6 and 8) a mild grade-II response (Figure 5, A). There was no correlation between the purity of CMV-CTLs after expansion and the skin histopathology grading (data not shown).

In clinical protocols, adoptive immunotherapy is considered feasible if virus-specific T-cells are matched to the recipient for at least one HLA-allele with confirmed anti-viral activity[39]. Shared HLA-alleles in donor-recipient pairs were analysed retrospectively by genotyping for 12 main HLA class-I and class-II alleles potentially presenting CMV-antigens. Three donor-recipient pairs from 9 experiments were matched respectively at HLA*A01:01, HLA*B07:02 and A*11:01, whereas no matches were found for the other donor-recipient pairs (Table 1). CMV-CTLs from donor 2, matched at HLA*A01:01 with recipient 2, did not show a positive GvHR. Similarly, CMV-CTLs from donor 5 matched at HLA*B07:02 with recipient 5. CMV-CTLs from donor 8 matched with recipient 8-b in A*11:01, but gave a positive grade-II GvHR as did the respective HLA-unmatched recipient 8-a. CMV-CTLs from donor 6 HLA-unmatched with recipient 6-a and 6-b showed a positive grade-II GvHR (data not shown). Together, these results showed no difference in the incidence of GvHR in the skin between HLA-virus restriction matched and unmatched donor-recipient pairs (Fisher's exact test: P=1.0) (SDC-Figure 5).

In addition, CMV-CTLs harvested from the skin explant assay exhibited significantly lower levels of CD25 expression compared to allo-unselected-PBMCs (P=0.00096, P=0.0489 and P=0.0299 for $CD3^+$, $CD4^+$ and $CD8^+$ T-cells, respectively) (n=4) (Figure 5, B).

To understand why CMV-CTLs led to reduced tissue damage in the skin explant assay, we investigated the repertoire of $CD4^+$ and $CD8^+$ T-cell subsets (n=4). In 2/4 CMV-CTLs, the CD4:CD8 ratio showed a bias towards the $CD8^+$ T-cell subset (81.77% and 79.10% for donor 5 and 3, respectively), whereas, the other 2 CMV-CTL lines (donor 6 and 8) exhibited a marked bias towards the $CD4^+$ T-cell subset (97.80% and 51.70%) (data not shown). As described previously, the CMV-CTLs from donor 6 and 8 yielded a higher GvHR-score (grade-II) at the $1x10^6$ cell-dose, thus suggesting a correlation between tissue damage and an increase of $CD4^+$ T-cells within the CMV-specific T-cells. The association between the frequency of the $CD4^+$ T-cells and the incidence of GvHR in the skin was investigated. As expected, the Spearman's test had a low power, due to n=4 (P=0.106), but a positive correlation coefficient (r_s=0.894) was observed, which corresponded to an increasing monotonic trend between the percentage of the $CD4^+$ T-cells and the incidence of GvHR (Figure 5-B+SDC-Figure 6).

CMV-CTLs secreted low levels of IFN-ϒ and granzyme B

To further support our experimental findings in the skin explant assay we measured IFN-ϒ and granzyme B in skin co-culture supernatants. The secretion of IFN-ϒ (Figure 6-A) and granzyme B (Figure 6-B) at both cell doses ($5x10^5$ and $1x10^6$ used in the skin explant assay) was significantly lower for CMV-CTLs compared to the corresponding cell dose of unselected-PBMCs from the same CMV^+donor. At the low and high cell dose, IFN-ϒ secretion by CMV-CTLs was found to be 4.16 [pg/ml] (±1.94 [pg/ml]) and 4.87 [pg/ml] (±4.50 [pg/ml]), respectively, while allo-unselected-PBMCs resulted in 139.80 [pg/ml] (±65.23 [pg/ml]) and 326.90 [pg/ml] (±201.3 [pg/ml]), respectively. The levels of granzyme B release were also significantly lower

at both cell doses, $5x10^5$ (*P*=0.033) and $1x10^6$ (*P*=0.0396), when compared to unselected-PBMCs.

Discussion

One major concern of using CMV-CTLs as an anti-viral therapy in the HLA-mismatched setting is that CMV-CTLs may recognize recipient HLA-antigens and induce alloreactive immune responses leading to GvHD. This study further elucidated the GvHR potential of HLA-mismatched third party CMV-CTLs using the unique human *in-vitro* skin explant assay[40-42]. We found that gamma-catch isolated CMV-CTLs possessed very low immunogenic capacity and gave rise to a reduced incidence of cutaneous GvH damage, whilst recognizing and killing target cells presenting the CMVpp65-antigen.

Our results differ from other *in-vitro* findings which have shown the cross-reactive potential of virus-specific T-cells and their possibility to induce GvHD[24-28]. This may be due to prolonged culture, cloning, repeated immuno-stimulation and different sets of growth factors, yielding to a different pattern of T-cells and probably contributing to the *in-vitro* alloreactivity.

Gamma-catch is based on the isolation of donor memory $CD4^+$ and $CD8^+$ T-cells responding specifically after short-term *in-vitro* stimulation by IFN-ϒ secretion. It is now applied to generate virus-specific T-cells under good manufacture practice conditions and has proved to be clinically effective by specifically clearing the viremia and restoring virus-specific immunity[13]. However, this cytokine-capture technology enriches not only T-cells, but any IFN-ϒ secreting cell, which could add to GvHD-onset/exacerbation. We therefore tested the use of this technology for the isolation of virus-CTLs and examined their capacity to induce GvHR.

After *in-vitro* expansion, highly enriched cell-lines were maintained, as demonstrated by intracellular IFN-ϒ detection following short-term re-stimulation with pp65-peptide.

Although the frequency of $CD3^+$ CMV-CTLs did not change after *in-vitro* expansion, the frequency of $CD4^+$ T-cells producing IFN-γ decreased to 16% upon re-stimulation. This may be due to several reasons, such as the use of different techniques for the IFN-γ detection pre and post-expansion or the production of other cytokines in response to antigen re-stimulation (e.g. TNF-α) from different $CD4^+$ T-cell subsets. However, this question requires further investigation.

The enriched and expanded CMV-CTLs could specifically kill pp65-loaded autologous PHA-blasts without inducing significant GvHR. This was supported by the significantly reduced allo-activation of CMV-CTLs compared to the allo-unselected-PBMCs in the MLRs, suggesting that CMV-CTLs may have a significantly lower risk of initiating GvHD.

To further analyse this, we established an *in-vitro* model predictive for GvHR by co-culturing a low ($5x10^5$) and a high ($1x10^6$) CMV-CTL numbers with skin. In clinical protocols, the number of adoptively transferred T-cells varies, depending on the donor and the yield obtained after isolation without further *in-vitro* expansion. Several clinical trials have reported efficient anti-viral activity independent from the T-cell dose transfused. Even very low cell numbers can successfully eradicate or markedly reduce the virus load[20,23,43,44]. In our *in-vitro* assay, both CMV-CTL doses ($5x10^5$ and $1x10^6$) showed a significant reduction in GvHR compared to PBMCs ($P<0.0006$ and $P<0.0001$). All CMV-CTLs tested at the $5x10^5$ T-cell doses showed a background reaction (grade-I GvHR) suggesting that CMV-CTLs may not react strongly against non-self HLA-molecules presented by haematopoietic cells and skin tissue. At $1x10^6$ T-cell doses, 2/9 CMV-CTL lines (donor 6 and 8) gave rise to a mild grade-II reaction suggesting an association between the cell dose and the severity of GvH-tissue damage (data not shown).

This is supported by significantly lower levels of IFN-Ɣ and granzyme B, both directly involved in skin damage[45], secreted by CMV-CTLs in the skin co-culture. Thus, we could conclude that short-term manipulated CMV-CTLs have low or no alloreactivity which was supported by the reduced CD25-activation in the $CD3^+$, $CD4^+$ and $CD8^+$ subsets in the skin explant (*P*=0.00096, *P*=0.0489 and *P*=0.0299). The use of additional activation markers may further consolidate the functionality of activated effector T-cells.

Analysing the composition of the CMV-CTLs showed that 2 lines contained more $CD4^+$ T-cells (97.80% and 51.70%) when compared to the two non-immunogenic CMV-CTL lines (9.39% and 2.79%). GvH-skin injury was induced (although at a low level) by the CTLs with higher $CD4^+$ content, thus suggesting an association between higher percentage of $CD4^+$ T-cells in the CMV-CTLs and the incidence of GvHR in the skin. This was supported by a positive correlation coefficient (*rs*=0.894) and supported by previous findings[46,47]. Especially, the $CD4^+$ central-memory and, to a lesser extent, the $CD4^+$ naïve T-cell subpopulations were associated with allo-immune responses, while effector-memory $CD4^+$ T-cells was shown to not raise immunogenic responses in HLA-identical sibling pairs[48]. A strategy to deplete allo-activated-$CD38^+CD4^{high}$ has been further proposed to reduce the incidence of GvHD while maintaining the non-immunogenic T-helper cells capable of reacting against pathogens and maintaining the functionality of $CD8^+$ T-cells[47]. Our data suggest that *in-vitro* expanded $CD4^+$ T-cells of the central-memory type, ($CD45RO^+CD62L^+$, data not shown), may be involved in the recognition of allo-antigens expressed by HLA-class-II alleles, but most importantly, that the frequency of these cells might be decisive in inducing GvH-skin damage.

The duality of virus-specific $CD4^+$ T-cells in potentially recognizing allo-HLA-antigens and in playing an essential role against viruses by maintaining the $CD8^+$ T-cell population[49,50] or directly fighting pathogens[51] needs further clarification. The transfusion of CMV-specific $CD8^+$ T-cells isolated by the streptamer technology, has been shown to successfully eradicate viral infection by *in-vivo* expansion and long-term persistence of donor $CD8^+$ T-cells, while simultaneously recruiting the local $CD4^+$ population, without signs of GvHD[52]. Others have reported that CMV-specific $CD4^+$ T-helper cells were essential for long-term persistence of transferred donor $CD8^+$ T-cell clones[11,53]. Furthermore, transfused $CD4^+$ T-cell lines generated by repetitive CMV-antigen pulsation of dendritic cells and short *in-vitro* expansion were able to efficiently restore anti-viral immunity in 5/7 patients lacking CMV-specific T-helper cells post-HSCT[54]. Clinical benefits were also observed in patients with refractory CMV, EBV or adenovirus infections, transfused with third-party multi-virus-specific T-cell lines. These results showed a marked heterogeneity in the composition of $CD4^+$ or $CD8^+$ CTLs, but concomitantly a very low incidence of GvHD[39].

Taken together, our results and those of others, suggest that the critical role of virus-specific $CD4^+$ T-cell lines for alloreactivity, particularly in the HLA-mismatched transplantation setting, requires further investigation. Patients showing a deficit of both cell types may profit from the establishment of an optimal, effective and safe $CD4^+$:$CD8^+$ ratio.

Here, we examined the alloreactive potential of CMV-CTLs specifically responding to pp65, which together with immediate-early protein IE-1, represent the two immunodominant antigenic target-proteins for CMV. Considering the importance of IE-1 to overcome and control CMV-infections[55], the dominating role of $CD4^+$ anti-IE1

proliferative responses[56] and the vast repertoire of IE-1 $CD4^+$ CTLs[57], testing IE-1-specific $CD4^+$ T-cells for alloreactivity would further clarify the safety of CMV-CTLs and the effectiveness of anti-CMV immunotherapy by targeting multiple-epitopes.

In conclusion, our pre-clinical data suggest that HLA-mismatched third party CMV-CTLs isolated by IFN-ϒ secretion assay may effectively reduce virus infected cells without inducing significant GvH-tissue damage, particularly when used at a low dose. However, the magnitude of the $CD4^+$ T-cell subsets within the transfused CMV-CTLs and their potential to induce GvHD, needs further elucidation. The human *in-vitro* GvHD model may be of value to evaluate the risk of GvHD induction prior to infusion of CMV-CTLs.

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HLA-Matches: Donor — Recipient	HLA Viral epitopes	A*01:01 pp65/pp50	A*02:01 pp65	A*11:01 pp65	A*24:02 pp65	B*07:02 pp65	B*44:03 pp65	B*44:02 pp65	B*40:01 pp65	B*35:01 pp65	B*35:02 pp65	B*08:01 IE-1	DRB1*01:01 pp65
	Recipient 1 ≠					X	X		X	X			
	Donor 1 ≠		X										
	Recipient 2 =	**X**	X							X	X		
	Donor 2 =	**X**			X							X	X
	Recipient 3 ≠							NA					
	Donor 3 ≠												
	Recipient 4 ≠							NA					
	Donor 4 ≠												
	Recipient 5 =			X		**X**		X					
	Donor 5 =					**X**	X						
	Recipient 6 a ≠							NA					
	Donor 6* ≠ / ≠	X										X	X
	Recipient 6 b ≠				X								
	Recipient 7 ≠		X										X
	Donor 7 ≠						X						
	Recipient 8 a ≠								X				
	Donor 8* ≠ / =			**X**		**X**						X	
	Recipient 8 b =	X	X	**X**		**X**		X					
	Recipient 9 ≠			X		X		X					
	Donor 9 ≠						X			X			X

≠HLA-unmatched; =HLA-matched; NA screening results not available. * Donor 6 was paired with recipient 6-a and 6-b. * Donor 8 was paired with recipient 8-a and 8-b.

Table 1: Matches between HLA-alleles potentially presenting CMV-epitopes in donor-recipient pairs

Figure 1: Scheme of the skin explant assay.

Figure 2 A: Percentage of IFN-γ^+ T-cells after stimulation with CMVpp65-antigen and isolation by the gamma-catch. Data are expressed as mean (±SEM) of twenty independent experiments (n=20). **B**: *In-vitro* expansion of CMV-CTLs after isolation using the gamma-catch. The graph represents CMV-CTL lines expanded with IL-2 and autologous irradiated feeder cells for 2 to 4 weeks for each individual independent experiment (n=14). CMV-CTLs were expanded from a mean (±SEM) of $3.71x10^5$ ($±9.55x10^4$) at isolation day (day 0) to $3.95x10^8$ ($±7.61x10^7$) after 15-28 days in culture. **C**: Absolute numbers of CMV-CTL populations after *in-vitro* expansion for 2 to 4 weeks. Horizontal bars indicate the mean (±SEM) values of fourteen independent CMV-CTL lines (n=14). ***P*=0.004.

Figure 3 Specificity of CMVpp65-CTLs after *in-vitro* expansion. A: Percentage of IFN-γ^+ T-cells after short-term re-stimulation with pp65 of expanded T-cells. The detection of IFN-γ was performed by intracellular staining and subsequent FACS analysis. Data are expressed as mean (±SEM) of fourteen independent experiments (n=14). **B**: Antigen-specific cytotoxicity of CMVpp65-CTLs after *in-vitro* expansion between 2 and 4 weeks. The graph represents the specific lysis of autologous PHA-blasts loaded and unloaded with pp65 at three different ratios, 20:1 [loaded= 93.59% (±1.33%); unloaded= 12.04% (±4.52%)], 5:1 [loaded= 81.10% (±8.72%); unloaded= 19.61% (±9.71%)] and 1:1 [loaded= 50.69% (±7.80%); unloaded= 10.85% (±5.91%)], between CMV-CTLs (effector) and PHA-blasts (target) in three independent experiments (n=3). ***P*=0.0079, **P*=0.0459.

Figure 4 CD25 expression of CMV-CTLs after the MLR. Percentage of $CD25^+$ T-cells in the autologous control: 11.04% (±2.00%) for $CD3^+$, 17.70% (±1.50%) for $CD4^+$ and 10.71% (±4.77%) for $CD8^+$ T-cells; allogeneic unselected-PBMCs: 66.35% (±3.64%) for $CD3^+$, 62.17% (±3.94%) for $CD4^+$ and 61.94% (±7.07%) for $CD8^+$ T-cells and allogeneic CMV-CTLs: 17.17% (±5.90%) for $CD3^+$, 38.20% (±5.50%) for $CD4^+$ and 17.59% (±4.43%) for $CD8^+$ T-cells. Data are expressed as mean (±SEM) of five independent experiments (n=5). Experiments were performed in duplicate where possible. ***P<0.0001, **P<0.004, ***P<0.0002.

Figure 5 A: Assessment of the alloreactive potential of CMV-CTLs using the skin explant assay. *Left*: Histopathological grade of nine independent experiments (n=9); skin sections were performed in duplicate when possible. Horizontal bars indicate the mean ±SEM values. P<0.0006 was calculated by *one-sample student t test* which compares the mean ±SEM of the histopathology grade of alloreactive unselected PBMCs with the GvHD grade-I of CMV-CTL lines tested at the low cell dose of $5x10^5$. ***P<0.0001. *Right*: Representative histological staining. Skin biopsies were co-cultured with A) medium only: grade-I; B) autologous-PBMCs: grade-I; C) $5X10^5$ unselected-PBMCs: grade-II; d) $5X10^5$ CMV-CTLs: grade-I; e) $1X10^6$ unselected-PBMCs: grade-III; F) $1X10^6$ CMV-CTLs: grade-I. Images were taken by Zeiss Axio Imager 2 microscope. Scale bar= 50µm. **B: CD25 activation and CMV-specific T-cell populations after the skin explant assay.** *Left:* Percentage of CD25 positive T-cells in the autologous control: 25.89% (±5.71%) for $CD3^+$, 33.48% (±5.65%) for $CD4^+$ and 10.27% (±4.84%) for $CD8^+$ T-cells; allogeneic-unselected-PBMCs: 68.45% (±6.05%) for $CD3^+$, 60.97% (±6.92%) for $CD4^+$ and 66.19% (±8.87%) for $CD8^+$ T-cells and allogeneic-CMV-CTLs: 32.92% (±7.32%) for $CD3^+$, 42.12% (±3.27%) for $CD4^+$ and 34.76% (±6.67%) for $CD8^+$ T-cells. Data are

expressed as mean (±SEM) of four independent experiments (n=4). Experiments were performed in duplicate when possible. ****P*<0.0001. *Right*: Spearman rank correlation between the percentages of the $CD4^+$ T-cell population in donors 3, 5, 6 and 8 after the skin explant assay and the incidence of GvHR (n=4).

Figure 6 Levels of IFN-ϒ and granzyme B released in the supernatants after co-culture of cells with skin sections. A: Comparison of IFN-ϒ release between unselected-PBMCs at $5x10^5$ and $1x10^6$ cell doses and CMV-CTLs at $5x10^5$ and $1x10^6$ cell doses. Data are expressed as mean ±SEM of six independent experiments (n=6). **B**: Comparison of granzyme B release between unselected-PBMCs ($5x10^5$; $1x10^6$) and CMV-CTLs ($5x10^5$; $1x10^6$), respectively 599.90 [pg/ml] (±208.10 [pg/ml]); 1048.00 [pg/ml] (±372.00 [pg/ml]) and 110.50 [pg/ml] (±59.03 [pg/ml]); 185.50 [pg/ml] (±138.40 [pg/ml]). Data are expressed as mean ±SEM of six independent experiments (n=6), **P*=0.0330, **P*=0.0396.

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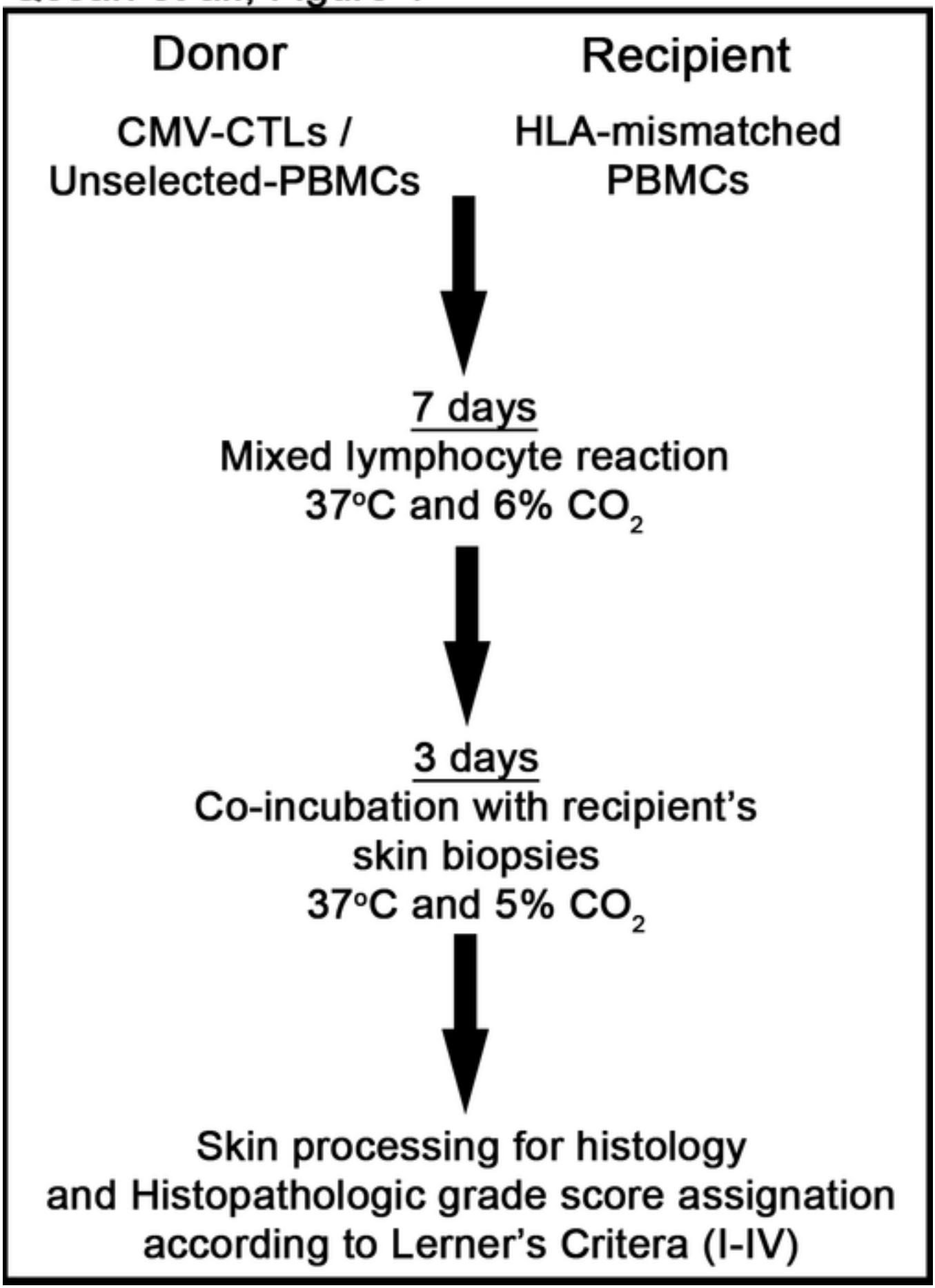
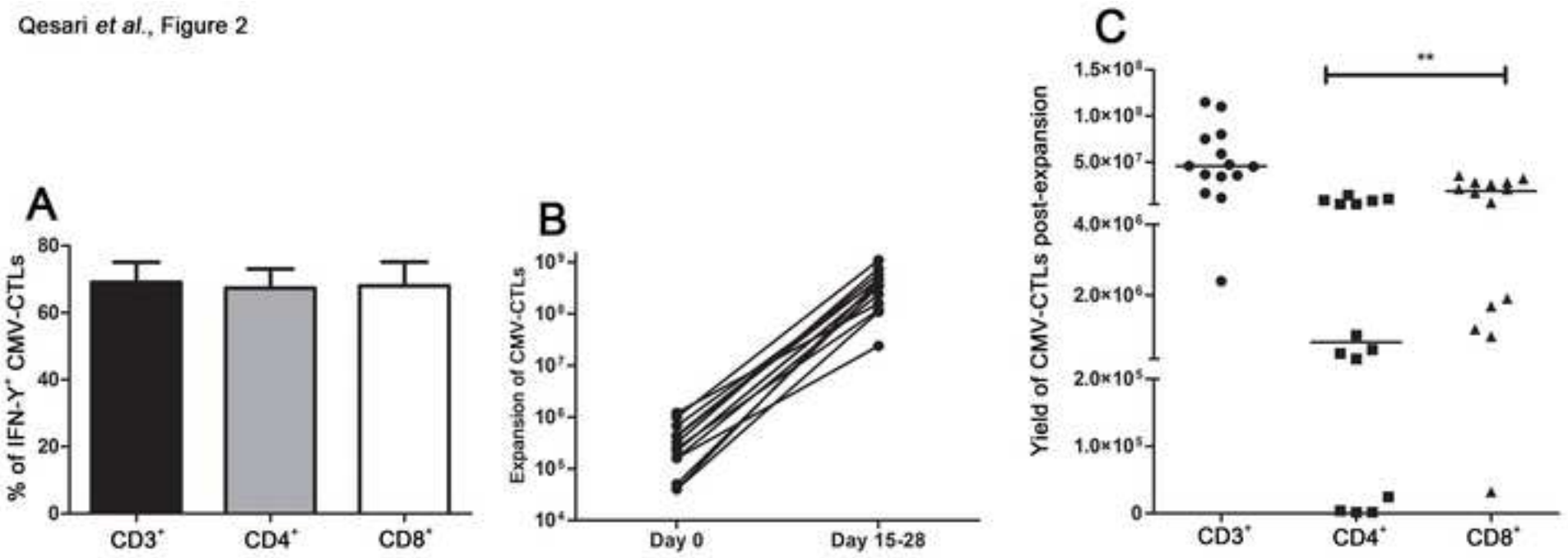
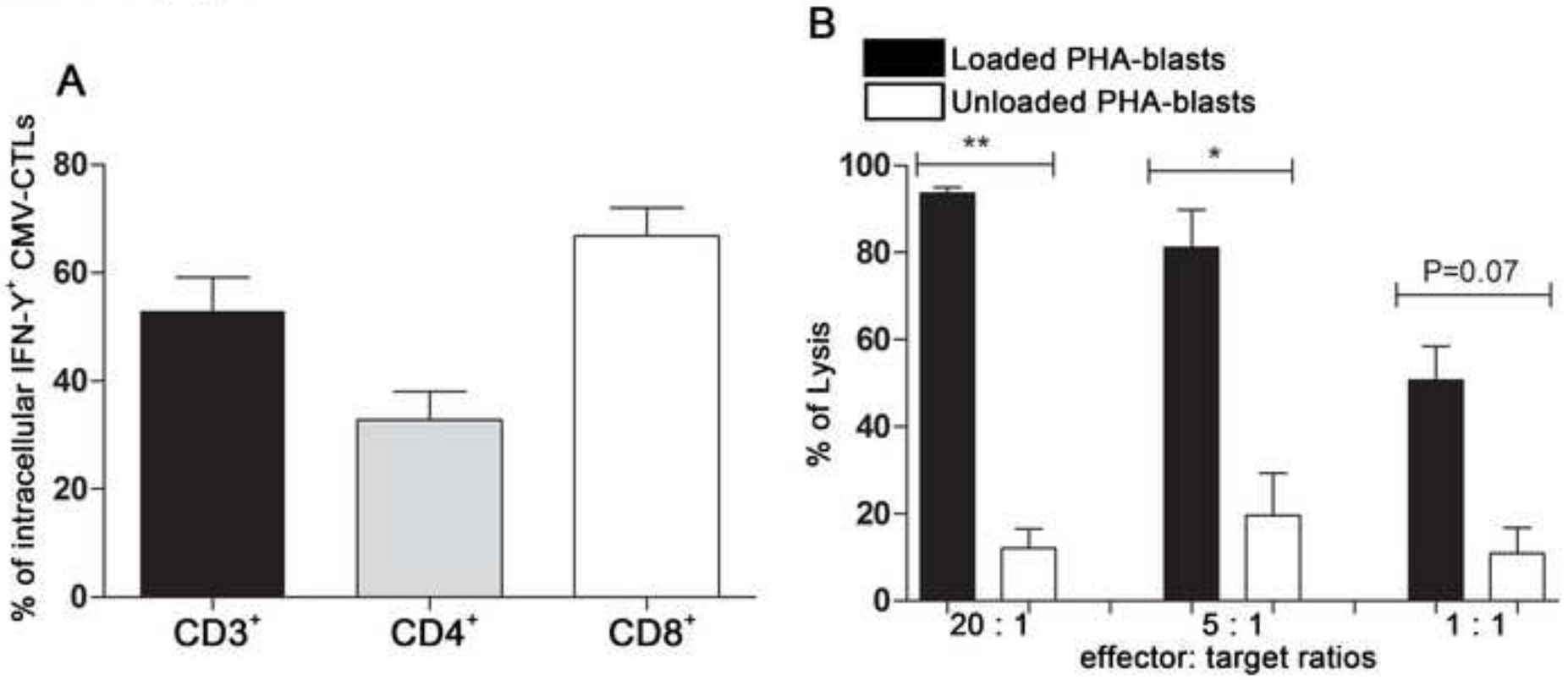


Qesari *et al.*, Figure 2



Qesari *et al.*, Figure 3



Qesari *et al.*, Figure 4

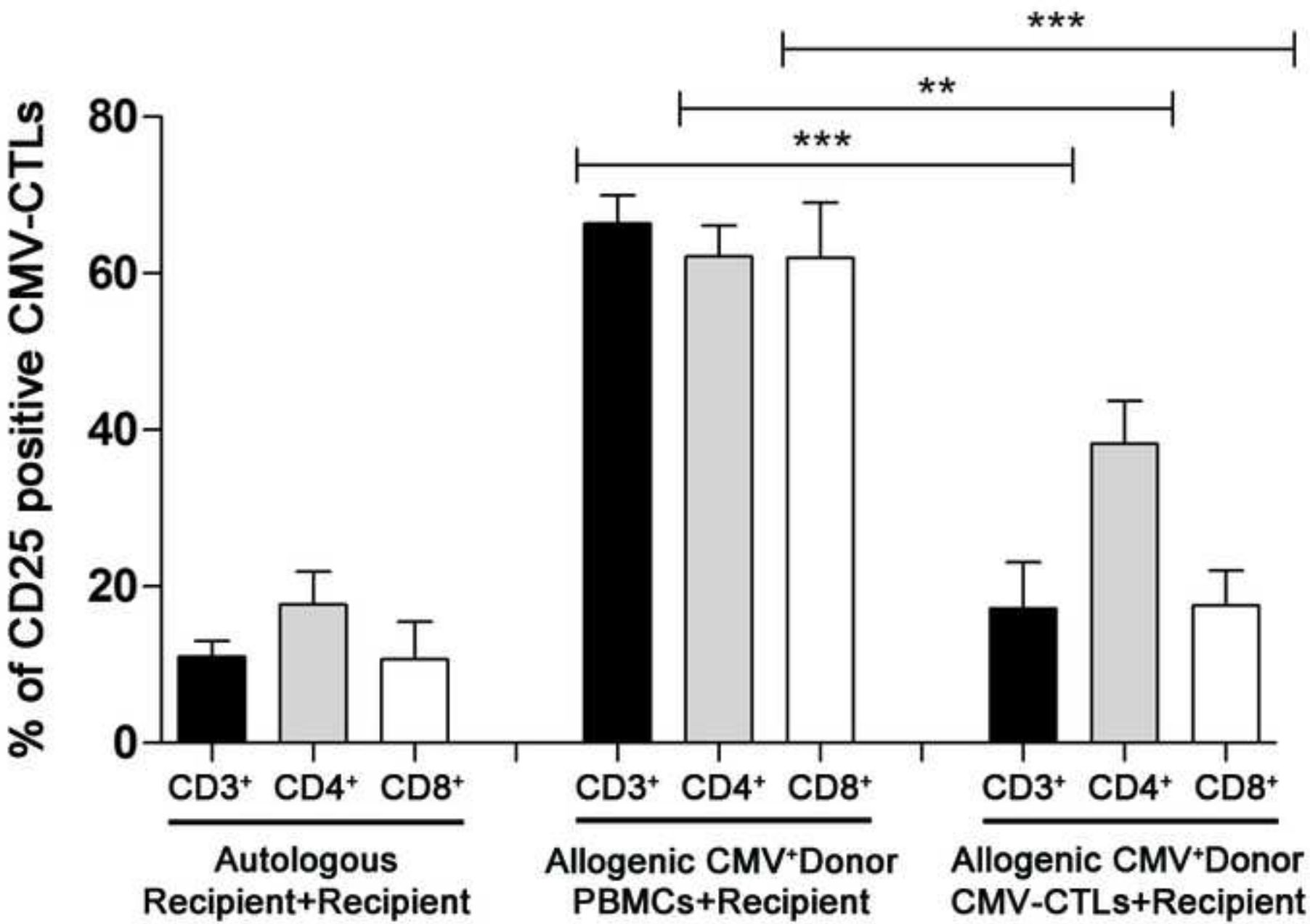


Figure5

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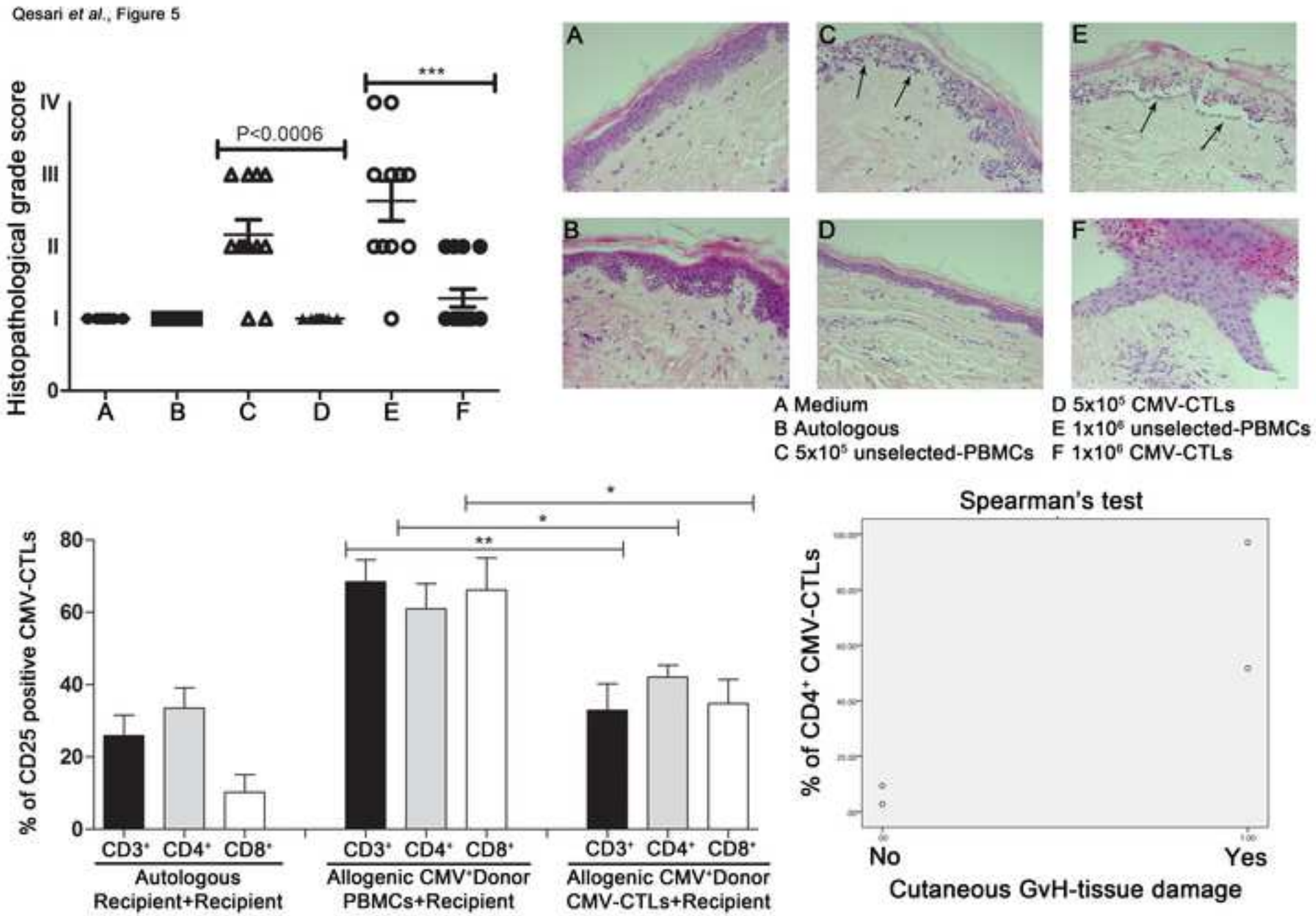

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